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Mammalian Target of Rapamycin-dependent Phosphorylation of PHAS-I in Four (S/T)P Sites Detected by Phospho-specific Antibodies*

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The role and control of the four rapamycin-sensitive phosphorylation sites that govern the association of PHAS-I with the mRNA cap-binding protein, eukaryotic initiation factor 4E (eIF4E), were investigated by using newly developed phospho-specific antibodies. Thr(P)-36/45 antibodies reacted with all three forms of PHAS-I that were resolved when cell extracts were subjected to SDS-polyacrylamide gel electrophoresis. Thr(P)-69 antibodies bound the forms of intermediate and lowest mobility, and Ser(P)-64 antibodies reacted only with the lowest mobility form. A portion of PHAS-I that copurified with eIF4E reacted with Thr(P)-36/45 and Thr(P)-69 antibodies but not with Ser(P)-64 antibodies. Insulin and/or amino acids increased, and rapamycin decreased, the reactivity of all three antibodies with PHAS-I in both HEK293 cells and 3T3-L1 adipocytes. Immunoprecipitated epitope-tagged mammalian target of rapamycin (mTOR) phosphorylated Thr-36/45. mTOR also phosphorylated Thr-69 and Ser-64 but only when purified immune complexes were incubated with the activating antibody, mTAb1. Interestingly, the phosphorylation of Thr-69 and Ser-64 was much more sensitive to inhibition by rapamycin-FKBP12 than the phosphorylation of Thr-36/45, and the phosphorylation of Ser-64 by mTOR was facilitated by phosphorylation of Thr-36, Thr-45, and Thr-69. In these respects the phosphorylation of PHAS-I by mTOR *in vitro* resembles the ordered phosphorylation of PHAS-I in cells.

PHAS-I (also known as 4E-BP1) is a key element in a regulatory system that governs translation initiation by controlling the availability of eIF4E[1] (1, 2). One of the first steps in initiation involves binding of eIF4E to the m^7GpppN (where N is any nucleotide) cap, which is found at the end of almost all eukaryotic mRNAs (3, 4). eIF4E also binds to eIF4G, a scaffolding protein that organizes several other important initiation factors, including eIF3, which links the complex to the 40 S ribosomal subunit. Nonphosphorylated PHAS-I binds tightly to eIF4E (5, 6), preventing its association with eIF4G (7, 8). When phosphorylated in the appropriate sites PHAS-I dissociates (5, 6), allowing eIF4E to engage eIF4G to form the complex that facilitates initiation.

PHAS-I in adipocytes and HEK293 cells is phosphorylated in the following five sites, all of which conform to a (S/T)P motif (9, 10): Thr-36, Thr-45, Ser-64, Thr-69, and Ser-82. Thr-45 and Ser-64 flank the eIF4E-binding motif (7, 8), and phosphorylation of either site blocks eIF4E binding *in vitro* (10, 11). Insulin stimulates the phosphorylation of Thr-36, Thr-45, Ser-64, and Thr-69 in both fat cells and HEK293 cells, and incubating cells with rapamycin decreases the phosphorylation of these sites (9, 10, 12). Determining which sites are most important in controlling PHAS-I in cells is complicated by the existence of an ordered mechanism in which the three TP sites are phosphorylated before phosphate accumulates in Ser-64 (10, 12).

The kinases participating in the ordered phosphorylation of PHAS-I are not known, although it is clear that the phosphorylation of PHAS-I in cells is controlled in part by the mTOR signaling pathway (1). mTOR is a member of the family of protein kinases that have catalytic domains more homologous to phosphatidylinositol 3-OH-kinase than to members of the much larger protein kinase family, of which the catalytic subunit of cAMP-dependent protein kinase is the prototypic member (13). The finding that rapamycin attenuated the phosphorylation of PHAS-I in response to insulin was the first indication that mTOR participated in the control of PHAS-I (14). Rapamycin is a potent and selective inhibitor of the function of mTOR in cells, although adding rapamycin directly to mTOR has little effect, as it is rapamycin complexed to FKBP12 that binds with high affinity to mTOR (15). FKBP12 also binds FK506 but forms a complex that does not bind to mTOR. Overexpressing mTOR increases PHAS-I phosphorylation in cells (16–18). Phosphorylation of PHAS-I becomes insensitive to rapamycin following expression of mTOR having a point mutation that disrupts high affinity binding to rapamycin-FKBP12. mTOR rendered kinase-dead by a point mutation in the kinase domain is ineffective in stimulating PHAS-I phosphorylation (16).

PHAS-I was the first exogenous substrate found for the mTOR kinase *in vitro* (16, 17). Based on peptide mapping and Edman degradation analyses of PHAS-I phosphorylated by mTOR that had been immunoprecipitated from rat brain ex-

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[1] The abbreviations used are: eIF3, eIF4E, and eIF4G, eukaryotic initiation factors 3, 4E and 4G, respectively; FKBP12, FK506-binding protein of M_r = 12,000; HEK, human embryonic kidney; [His^6]PHAS-I, histidine-tagged PHAS-I; mTAb1 and mTAb2, mTOR antibodies 1 and 2, respectively; mTOR, mammalian target of rapamycin; PAGE, polyacrylamide gel electrophoresis; WT, wild type; MAP, mitogen-activated protein.

tracts with the antibody, mTAb1, it was concluded that mTOR was capable of phosphorylating Thr-36, Thr-45, Thr-69, and Ser-64 (19). Subsequent studies by other groups failed to confirm the phosphorylation of Ser-64 and Thr-69, leading to suggestion that phosphorylation of PHAS-I by mTOR is confined to Thr-36 and Thr-45 (12, 17). However, different antibodies were used to immunoprecipitate mTOR, an important point in view of the fact that mTAb1 is an activating antibody (19).

Most previous studies of the phosphorylation of PHAS-I in cells have relied upon measurements of the gel shift that PHAS-I undergoes when it is phosphorylated in the appropriate sites. Three forms, designated α, β, and γ in order of decreasing electrophoretic mobility, are typically resolved by SDS-PAGE (20). Our studies have demonstrated that Thr-45 and Ser-64 may be phosphorylated without a significant change in electrophoretic mobility (10, 11). Moreover, because more than one combination of sites may be phosphorylated in each mobility form, the gel shift assay provides no definitive information on the phosphorylation state of specific sites in PHAS-I (10). To address this problem, we have generated a series of phospho-specific antibodies to sites in the PHAS-I protein. In this report, we present evidence documenting the specificity of the antibodies, and we describe results that provide new insight into the role of sites in controlling eIF4E binding as well as the mechanisms involved in the ordered phosphorylation of PHAS-I by the mTOR signaling pathway.

EXPERIMENTAL PROCEDURES

Antibodies—The mTOR antibodies, mTAb1 and mTAb2, were described previously (19). PHAS-I antibodies were generated by immunizing rabbits with a synthetic peptide (CSSPEDKRAGGEESQFE) as described previously (21). These antibodies bind in the COOH-terminal region of PHAS-I and react equally well with phosphorylated and nonphosphorylated forms of the protein. To generate antibodies that specifically recognize PHAS-I phosphorylated in Thr-36/Thr-45, Ser-64, or Thr-69, rabbits were immunized with the following phosphopeptides coupled to keyhole limpet hemocyanin: Thr(P)-36/45, CGDYSTT*PGGT; Ser(P)-64, CRNS*PVAK; and Thr(P)-69, CNSPVAKT*PPKD (where S* and T* are phosphorylated Ser and Thr, respectively). Note that the amino acid sequence surrounding the Thr-36 site (YSTT*PGGT) is almost identical to that surrounding the Thr-45 site (FSTT*PGGT) (9), so that phospho-specific antibodies generated against the Thr-36/45 peptide would be expected to react with PHAS-I when either Thr-36 or Thr-45 is phosphorylated. To select the phospho-specific antibodies, the serum was first incubated with nonphosphorylated peptides (CGDYSTTPGGTLFSTTPGGTR for Thr(P)-36/45, CRNSPVAKTPPKDLPT, for Ser(P)-64 and Thr(P)-69) that had been coupled to SulfoLink resin (Pierce). Next, the fractions that did not bind to the nonphosphorylated peptide resins were incubated with the respective phosphopeptides coupled to SulfoLink. After washing the columns, the phospho-specific antibodies were eluted at pH 2.7, immediately neutralized, and affinity-purified using protein A-agarose.

Preparation of Recombinant Proteins—Wild type (WT) PHAS-I and PHAS-I proteins having Ser/Thr → Ala mutations at positions 36, 45, 64, and 69 were expressed in bacteria and purified as described by Yang *et al.* (11). Designations for various mutant PHAS-I proteins are as described by Mothe-Satney *et al.* (10). Histidine-tagged PHAS-I ([His^6]PHAS-I) was prepared as described previously (22). [His^6]S64 PHAS-I was generated by inserting cDNA encoding S64 PHAS-I into pET14b (Novagen) and expressing the protein in bacteria. Recombinant glutathione *S*-transferase-FKBP12 was purified as described previously (23). Recombinant MAP kinase was purified and activated as described by Fadden *et al.* (9).

Cell Culture and Transfections—HEK293 cells (ATCC, CRL 1573) were seeded into plastic tissue culture dishes (Falcon, 2×10^4 cells/cm^2) and cultured in a humidified atmosphere of 5% CO_2 in air for 24 h in growth medium composed of 10% (v/v) horse serum in Dulbecco's modified Eagle's medium. The construction of pCMV4 vectors for the expression of WT PHAS-I and mutant PHAS-I proteins and the procedures used in transfecting HEK293 cells were described previously (10).

293T cells were transfected with pcDNA3 alone or pcDNA3 containing inserts encoding AU1-mTOR proteins by using TransIT-LT2 polyamine transfection reagent (PanVera Corp., Madison, WI) and 5 μg of DNA/100-mm diameter dish as described previously (16). AU1-mTOR has an AU1 epitope tag at the NH_2 terminus. AU1-mTOR (rr) and AU1-mTOR (kd) have Ser-2035 → Ile and Asp2338 → Ala mutations, respectively.

3T3-L1 adipocytes were cultured in 10-cm diameter dishes and used in experiments 8–10 days after removal of the differentiation medium. Cells were incubated at 37 °C for 2.5 h in HEPES-buffered saline (145 mM NaCl, 5.4 mM KCl, 1.4 mM $CaCl_2$, 1.4 mM $MgCl_2$, 0.1 mM NaP_i, 5 mM glucose, 0.5% bovine serum albumin, and 10 mM Na-HEPES, pH 7.4) and then incubated as indicated in Fig. 4. Extracts were prepared as described previously (14).

Immune Complex Assay of mTOR Kinase—HEK293T cells that had been transfected with vector alone or with the pcDNA-3 AU1-mTOR constructs were cultured for 18 h, washed twice with Buffer A (150 mM NaCl and 50 mM Tris-HCl, pH 7.4), and homogenized at 0 °C in Buffer B (50 mM NaF, 1 mM EDTA, 1 mM EGTA, 0.1% Tween 20, 10 mM potassium phosphate, and 50 mM β-glycerophosphate, pH 7.4) (750 μl/100-mm diameter dish) supplemented with 1 mM dithiothreitol, 2.5 mM $MgCl_2$, 0.5 μM microcystin LR, 0.5 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of leupeptin, pepstatin, and aprotinin. Homogenates were centrifuged at 10,000 × *g* for 30 min at 4 °C. mTOR was immunoprecipitated from aliquots (700 μl) of extract using anti-AU1 antibody (from 2 μl of ascites) (Babco, Richmond, CA) bound to protein G-agarose (15 μl of packed beads). After incubating with extracts for 180 min, the beads were washed (1 ml buffer/wash) at 4 °C as follows: twice in Buffer B, twice in buffer B plus 0.5 M NaCl, and twice in Buffer C (1 mM EDTA, 1 mM EGTA, and 50 mM Tris-HCl, pH 7.4). The beads were then incubated for 15 min at 4 °C in Buffer C and then washed twice in Buffer D (50 mM NaCl, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 μM microcystin LR, 10 mM Na-HEPES, and 50 mM β-glycerophosphate, pH 7.4). Prior to the kinase assay, the washed beads were incubated at 22 °C for 90 min in 25 μl of Buffer D supplemented with FKBP12, rapamycin, FK506, and mTOR antibodies. The beads were then washed twice with Buffer D, and the kinase reactions were initiated by adding 25 μl of Buffer D supplemented with 0.1 mM [γ-^{32}P]ATP (2000 mCi/mmol), 10 mM $MnCl_2$, and 50 μg/ml of the appropriate PHAS-I protein. The reactions were terminated by adding SDS sample buffer.

Immunoprecipitation of PHAS-I and Affinity Purification of PHAS-I·eIF4E Complexes with m^{7}GTP-Sepharose—PHAS-I was immunoprecipitated from cell extracts by using the antibody to the COOH-terminal region of the protein, and PHAS-I·eIF4E complexes were isolated from cell extracts by using m^{7}GTP-Sepharose beads as described previously (5).

Electrophoretic Analyses—Samples were subjected to SDS-PAGE by using the method of Laemmli (24). PHAS-I was identified by immunoblotting with PHAS-I antibodies as described previously (25). Binding of phospho-specific antibodies were assessed in the same manner.

RESULTS

Antibody Specificity—To assess the specificity of the antibodies used in this study, recombinant WT PHAS-I and mutant PHAS-I proteins having Ser/Thr to Ala mutations in different phosphorylation sites were phosphorylated *in vitro* with MAP kinase before samples were subjected to SDS-PAGE, and immunoblots were prepared with the different antibodies (Fig. 1). A36 PHAS-I, A45 PHAS-I, A64 PHAS-I, and A69 PHAS-I have Ser/Thr → Ala mutations in Thr-36, Thr-45, Ser-64, and Thr-69, respectively. A36/A45 PHAS-I has Thr → Ala mutations in both Thr-36 and Thr-45. MAP kinase is able to phosphorylate all four of these (S/T)P sites in WT PHAS-I, although Thr-69 is phosphorylated relatively slowly (9). All of the antibodies reacted with WT PHAS-I that had been phosphorylated with MAP kinase (Fig. 1). However, none of the phospho-specific antibodies reacted with any of the PHAS-I proteins that had not been incubated with MAP kinase,[2] indicating that recognition by the antibodies is dependent on phosphorylation. Results with the mutant proteins provided further support of the specificity of the antibodies. Thus, the Ser(P)-64 antibody bound to all of the phosphorylated PHAS-I proteins, except A64 PHAS-I, indicating that Ser-64 phosphorylation was required for recognition by this antibody. Likewise, mutating Thr-69 ablated binding by the Thr(P)-69 antibody. Mutating either Thr-36 or

[2] I. Mothe-Satney and J. C. Lawrence, Jr., unpublished observations.

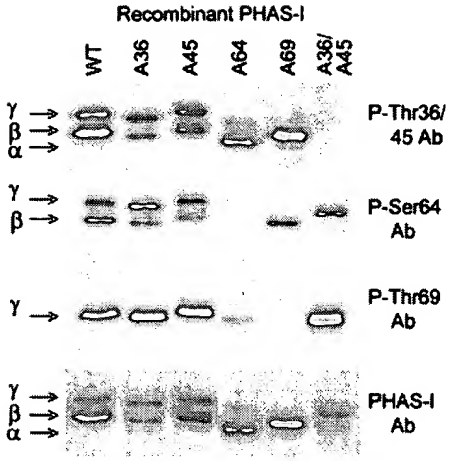

FIG. 1. **Reactivity of antibodies with WT PHAS-I and mutant PHAS-I proteins after *in vitro* phosphorylation with MAP kinase.** WT PHAS-I and PHAS-I proteins having Ser/Thr to Ala mutations in the four insulin-sensitive phosphorylation sites were incubated with activated MAP kinase and ATP for 5 h as described previously (10). Samples were then subjected to SDS-PAGE, and proteins were transferred to an Immobilon membrane. Immunoblots were prepared with Thr(P)-36/45 antibodies (*P-Thr36/45 Ab*), Ser(P)-64 antibodies (*P-Ser64 Ab*), Thr(P)-69 antibodies (*P-Thr69 Ab*), or PHAS-I antibodies. α, β, and γ denote electrophoretic mobility forms of PHAS-I.

Thr-45 decreased binding of the Thr(P)-36/45 antibody, and mutating both Thr-36 and Thr-45 was required to abolish binding of this antibody. These results indicate that Thr(P)-36/45 recognizes the phosphorylated forms of either Thr-36 or Thr-45.

To determine whether the phospho-specific antibodies could be used to investigate the phosphorylation of PHAS-I in intact cells, WT PHAS-I and mutant PHAS-I proteins were expressed in HEK293 cells, which were then incubated with a combination of insulin and amino acids to stimulate phosphorylation of the expressed proteins. When compensations are made for the slightly different amounts of the proteins expressed, as judged by blotting with PHAS-I antibodies, it can be seen that the Thr(P)-36/45 antibodies reacted equally well with WT PHAS-I, A64 PHAS-I, A69 PHAS-I, and A82 PHAS-I (Fig. 2*A*). Mutating either Thr-36 or Thr-45 decreased binding of the Thr(P)-36/45 antibodies, but mutation of both sites was required to abolish binding. The findings are consistent with the results from the *in vitro* phosphorylation experiments (Fig. 1) and indicate that the Thr(P)-36/45 antibodies bind to PHAS-I phosphorylated in either Thr-36 or Thr-45. Also consistent with the results from *in vitro* studies of specificity, Thr(P)-69 bound to all of the mutant proteins, except A69 PHAS-I (Fig. 2*A*).

Ser(P)-64 antibodies recognized WT PHAS-I in cell extracts of HEK293 cells, but other proteins having mobilities similar to that of PHAS-I reacted with the antibody.[2] For this reason, when using Ser(P)-64 antibodies to investigate the phosphorylation of PHAS-I in HEK293 cells, we immunoprecipitated PHAS-I before immunoblotting with Ser(P)-64 antibodies (Fig. 2*B*). As expected Ser(P)-64 antibodies also bound A82 PHAS-I but did not bind to PHAS-I protein having a Ser-64 → Ala mutation. In addition, little if any binding of Ser(P)-64 antibodies to A36 PHAS-I, A45 PHAS-I, or A69 PHAS-I was detected. These findings provide additional evidence that accumulation of phosphate in Ser-64 depends on the phosphorylation of all three TP sites.

Binding of eIF4E to PHAS-I markedly inhibits the phosphorylation of PHAS-I by certain kinases *in vitro* (14). To investigate the possibility that phosphorylation of Ser-64 occurred secondarily to dissociation of the PHAS-I·eIF4E complex in

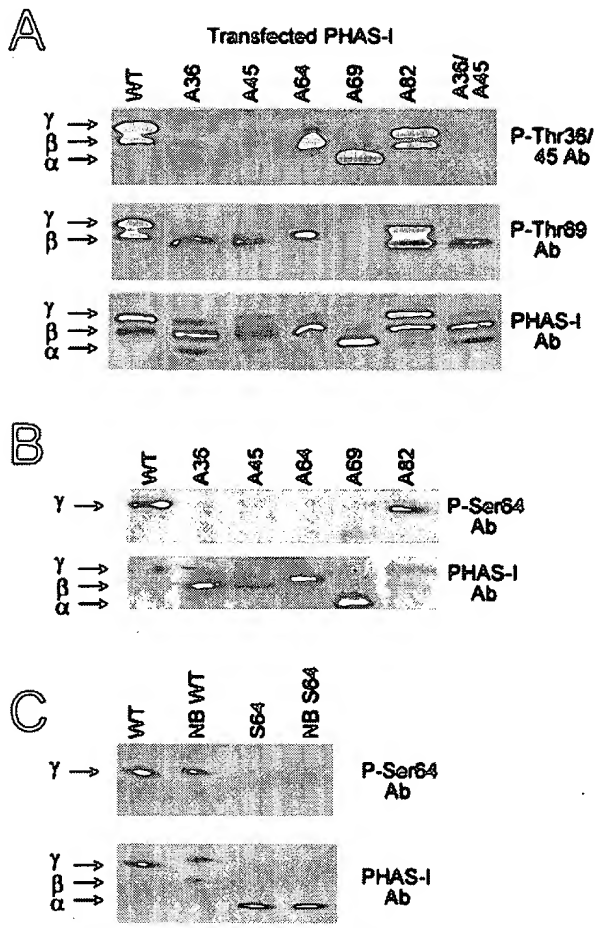

FIG. 2. **Reactivity of antibodies with WT PHAS-I and mutant PHAS-I proteins after expression in HEK293 cells.** Wild-type and mutant PHAS-I proteins were expressed in HEK293 cells, which were then stimulated with insulin for 15 h and lysed. *A*, extract samples were subjected to SDS-PAGE, and immunoblots were prepared with Thr(P)-36/45 antibodies, Thr(P)-69 antibodies, or PHAS-I antibodies. *B*, PHAS-I proteins were immunoprecipitated and then subjected to SDS-PAGE before immunoblots were prepared with Ser(P)-64 antibodies or PHAS-I antibodies. *C*, WT-PHAS-I, NB WT PHAS-I, S64 PHAS-I, and NB S64 PHAS-I were expressed in HEK293 cells, which were then incubated with insulin for 15 h. NB WT PHAS-I and NB S64 PHAS-I have mutations that prevent high affinity binding to eIF4E (10). The PHAS-I proteins were immunoprecipitated before immunoblots with Ser(P)-64 antibodies and with PHAS-I antibodies were prepared.

cells, we conducted experiments with NB PHAS-I, a protein with Leu-58 → Ala and Met-59 → Ala mutations, which have been shown to abolish high affinity binding of PHAS-I to eIF4E (10, 26). Results with Ser(P)-64 antibodies confirmed that Ser-64 was phosphorylated in both WT PHAS-I and NB PHAS-I (Fig. 2*C*). However, Ser-64 was phosphorylated in neither S64 PHAS-I nor NB S64 PHAS-I, proteins having Ser → Ala mutations in all of the sites except Ser-64. Thus, the dependence of Ser-64 phosphorylation on the other phosphorylation sites does not occur because of dissociation of the PHAS-I·eIF4E complex in response to phosphorylation of the TP sites.

Rapamycin-sensitive Control of PHAS-I Phosphorylation by Insulin and Amino Acids—We next used the phospho-specific antibodies to investigate control of the phosphorylation of different sites in HEK293 cells overexpressing WT PHAS-I. Incubating cells with either insulin or amino acids alone increased Thr(P)-36/45 binding by approximately 3-fold (Fig. 3*A*). Rapamycin had relatively little effect on the basal level of Thr(P)-36/45 binding, but rapamycin essentially abolished the effects

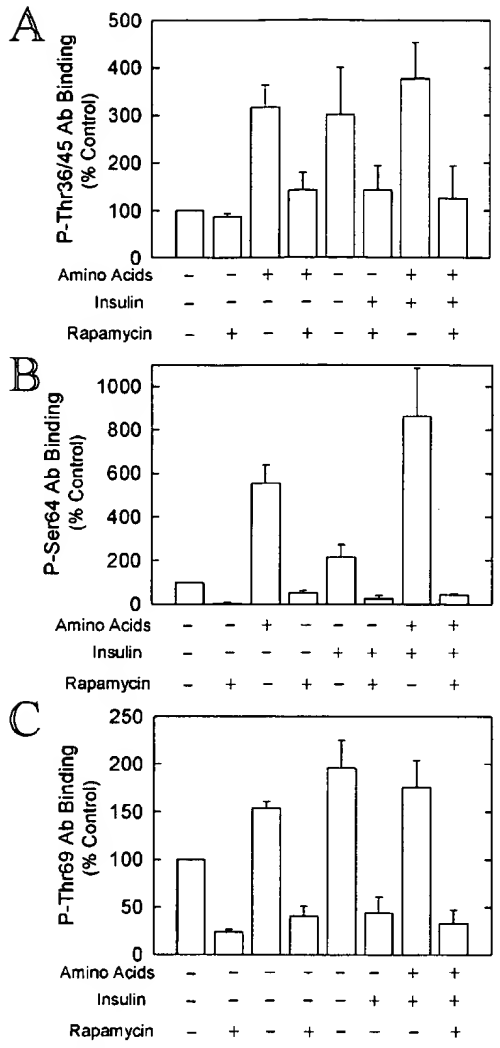

FIG. 3. **Phospho-specific antibody immunoblots of PHAS-I from HEK293 cells incubated with amino acids and insulin.** WT PHAS-I was overexpressed in HEK293 cells, which were then placed in medium without serum or amino acids and incubated at 37 °C for 3 h before extracts were prepared. Where indicated, rapamycin (50 nM) was added after 90 min. Recombinant human insulin (700 nM) and/or amino acids (25-fold dilution of a 50× minimum Eagle's medium amino acid mixture) were added after 150 min. Immunoblots were prepared, and relative intensities of the bands of phosphorylated PHAS-I were determined by optical density scanning. After correcting for total PHAS-I, which was determined from PHAS-I blots, the results were expressed as percentages of the respective controls. Results for Thr(P)-36/45 antibody binding (*A*) and Thr(P)-69 antibody binding (*C*) are means + S.E. from three experiments. Results for Ser(P)-64 antibody binding (*B*) are means + ½ range of two experiments.

of insulin and amino acids. Insulin and amino acids increased the binding of Ser(P)-64 antibodies by approximately 2- and 5-fold, respectively, and the effects were approximately additive (Fig. 3*B*). Insulin also increased binding of Thr(P)-69 by 2-fold, but the effects of amino acids on Thr(P)-69 binding (Fig. 3*C*) were less than the effects on Ser(P)-64 binding (Fig. 3*B*). Incubating cells with rapamycin dramatically decreased binding of Ser(P)-64 and Thr(P)-69, both in cells incubated without other additions and in cells incubated with insulin and/or amino acids (Fig. 3, *B* and *C*).

Incubating 3T3-L1 adipocytes with insulin increased the reactivity of PHAS-I with the three phospho-specific antibodies, although the effects of insulin were less in 3T3-L1 adipocytes (Fig. 4, *A–C*) than in HEK293 cells (Fig. 3, *A–C*). Rapamycin decreased reactivity with the different antibodies and attenuated the effects of insulin in 3T3-L1 adipocytes (Fig. 4, *A–C*), but the effects of rapamycin were also less pronounced in the adipocytes.

Although phosphorylation of PHAS-I is known to promote dissociation of the PHAS-I·eIF4E complex, certain phosphorylated forms of PHAS-I may copurify with eIF4E. An implication is that phosphorylation of the sites in such forms is not sufficient to promote dissociation of the PHAS-I·eIF4E complex. Therefore, to gain further insight into the sites controlling PHAS-I binding to eIF4E in cells, PHAS-I·eIF4E complexes were affinity-purified from extracts of 3T3-L1 adipocytes (Fig. 5, *A* and *B*). In samples of the affinity-purified preparations from control cells, bands corresponding to α and β forms were detected with PHAS-I antibodies. The α form from control cells did not exhibit reactivity with any of the three phospho-specific antibodies, consistent with the interpretation that the band was composed primarily of nonphosphorylated PHAS-I. PHAS-I in the β band reacted with Thr(P)-69 antibodies (Fig. 5*B*). Incubating cells with insulin essentially abolished binding of PHAS-I to eIF4E, as evidenced by the nearly complete loss of PHAS-I immunoreactivity in the m^7GTP-Sepharose-purified fraction. Rapamycin attenuated the effect of insulin, and after treatment with rapamycin, phosphorylated Thr-69 was once again detected in the pool of PHAS-I bound to eIF4E. Moreover, after rapamycin, reactivity with Thr(P)-36/45 was detected in the α band. Phosphorylated Ser-64 was not detected in the fraction of PHAS-I that copurified with eIF4E.

Phosphorylation of PHAS-I by mTOR—To investigate the sites in PHAS-I phosphorylated by mTOR, an AU1 epitope-tagged mTOR was overexpressed in HEK293T cells. mTOR was immunoprecipitated by using an AU1 antibody, and the ability of the preparation to phosphorylate recombinant PHAS-I was investigated after exhaustively washing the immune complexes. None of the phospho-specific antibodies reacted with PHAS-I that had been incubated with immune complexes derived from cells that had been transfected with the pcDNA3 vector alone (Fig. 6, *A–C*). Incubating PHAS-I with mTOR immune complexes markedly increased reactivity with the Thr(P)-36/45 antibodies (Fig. 6*A*, *middle panel*). As would be expected of an mTOR-mediated process, incubating immune complexes with rapamycin or FKBP12 alone did not attenuate the phosphorylation, but it was markedly inhibited when the mTOR preparation was incubated with the combination of rapamycin plus FKBP12. In contrast, incubation with FK506 plus FKBP12 did not inhibit the phosphorylation of PHAS-I. The reactivity of Thr(P)-36/45 was increased severalfold when the immunopurified mTOR was incubated with mTAb1, indicating that the activating antibody increases the rate at which mTOR phosphorylates Thr-36 and/or Thr-45 (Fig. 6*A*, *bottom panel*).[3] The effect of rapamycin-FKBP12 on decreasing reactivity with Thr(P)-36/45 was less pronounced after activation of mTOR with mTAb1.

Phosphorylation of Ser-64 was not detected when AU1-mTOR was incubated with PHAS-I in the absence of activating antibody (Fig. 6*B*, *middle panel*). However, reactivity with Ser(P)-64 antibodies was readily detected after immune complexes were incubated with mTAb1 (Fig. 6*B*, *bottom panel*). Phosphorylation of Ser-64 was abolished by FKBP12 plus rapamycin but was not inhibited by FKBP12 plus FK506. Thr-69

[3] After incubating mTOR with mTAb1, a band corresponding to PHAS-I β was observed. This band was most prominent in samples incubated with FK506. The increase in mTOR activity in response to FK506 has also been observed in samples of mTOR immunoprecipitated from rat brain extracts with the antibodies mTAb1 and mTAb2. The mechanism underlying this effect is unclear.

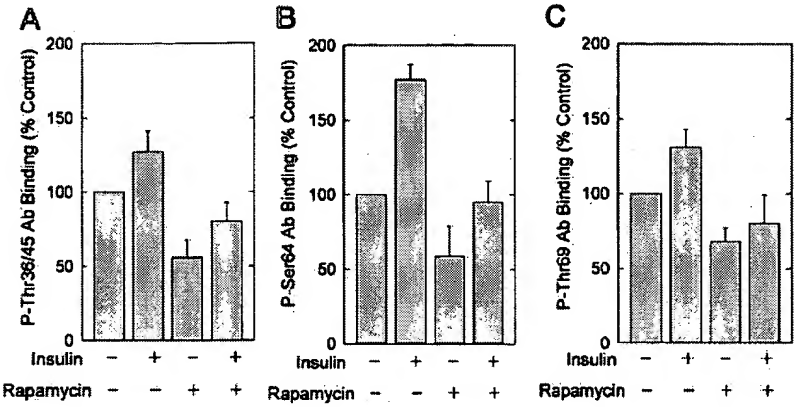

FIG. 4. **Effect of incubating 3T3-L1 adipocytes with insulin and rapamycin on phospho-specific antibody binding to PHAS-I.** 3T3-L1 adipocytes were incubated at 37 °C in HEPES-buffered saline for 150 min. The cells were then incubated without additions, with 50 nM rapamycin for 30 min, with 70 nM insulin for 10 min, or with rapamycin for 20 min and then insulin plus rapamycin for 10 min. Sample extracts were subjected to SDS-PAGE, and immunoblots were prepared with the different phospho-specific antibodies to PHAS-I. Relative intensities of the bands of phosphorylated PHAS-I were determined by optical density scanning. After correcting for total PHAS-I present, the results were expressed as percentages of the respective controls. Results for Thr(P)-36/45 antibody binding (*A*), Ser(P)-64 antibody binding (*B*), and Thr(P)-69 antibody binding (*C*) are means + S.E. from three experiments.

phosphorylation was also dramatically increased in response to the activating antibody (Fig. 6*C, bottom panel*). Phosphorylation of Thr-69 was almost completely inhibited by rapamycin plus FKPB12, but not by rapamycin, FK506, or FKBP12 alone.

To investigate further the phosphorylation of Thr-69 by mTOR, AU1-tagged mTOR was incubated with T69 PHAS-I in a solution containing Mn^{2+} and [γ-^{32}P]ATP (Fig. 7). T69 PHAS-I has Ser/Thr → Ala mutations in Thr-36, Thr-45, Ser-64, and Ser-82. Phosphorylation of T69 PHAS-I resulted in the appearance of a ^{32}P-labeled β form, indicative of Thr-69 phosphorylation, which we have shown promotes an α to β shift. No phosphorylation of T69 PHAS-I was detected in cells transfected with vector alone, demonstrating that phosphorylation of T69 PHAS-I was dependent on expression of AU1-mTOR. T69 PHAS-I phosphorylation was increased severalfold by mTAb1. mTAb2, an antibody that recognizes a different epitope in mTOR (19), did not affect the phosphorylation of T69 PHAS-I. Rapamycin plus FKBP12 markedly reduced ^{32}P incorporation into T69 PHAS-I, but the complex of FK506·FKBP12 did not inhibit phosphorylation.

T69 PHAS-I was also incubated with AU1-mTOR (rr) and AU1-mTOR (kd), proteins having a Ser-2035 → Ile and an Asp-2338 → Ala mutation, respectively. The point mutation at position 2035 results in a catalytically active mTOR that has a lower affinity for rapamycin-FKBP12 (27). T69 PHAS-I was phosphorylated by this mutant mTOR, but phosphorylation was not inhibited by rapamycin-FKBP12, consistent with the rapamycin-resistant properties of this form of mTOR (Fig. 7). The point mutation at position 2338 abolishes the phosphotransferase activity of mTOR (16, 27), and T69 PHAS-I was not phosphorylated by immune complexes containing this kinase-dead form of mTOR.

Phosphorylation of Ser-64 alone does not decrease the electrophoretic mobility of PHAS-I, and some of the phosphate incorporated into Ser-64 by the activated mTOR was associated with the α form (Fig. 6*B*). However, most of the Ser-64 phosphorylation catalyzed by mTOR appeared in the γ form, suggesting that mTOR prefers to phosphorylate Ser-64 after other sites have been phosphorylated. To investigate this hypothesis, we compared the phosphorylation of WT PHAS-I and S64 PHAS-I, a recombinant protein with Ser/Thr → Ala mutations in Thr-36, Thr-45, Thr-69, and Ser-82. Both proteins were phosphorylated in Ser-64 following incubation of immune complexes with mTAb1 (Fig. 8*A*); however, the phosphorylation of Ser-64 in WT PHAS-I was much higher than in S64 PHAS-I (Fig. 8*A*), even though equal amounts of the proteins were present (Fig. 8*B*).

DISCUSSION

Phospho-specific antibodies provide an alternative to ^{32}P labeling and peptide mapping for examining changes in the phosphorylation of the four rapamycin-sensitive (S/T)P sites in PHAS-I. The results with these antibodies further define the sites responsible for the electrophoretic mobility shift and draw attention to some of the serious limitations of the gel-shift assay in assessing phosphorylation of PHAS-I. More importantly, the results provide new insight into the sites responsible for the dissociation of the PHAS-I·eIF4E complex in cells and into the control of PHAS-I function by the mTOR signaling pathway.

Recent evidence suggests that the phosphorylation of PHAS-I occurs in an ordered fashion, in which the TP sites must be phosphorylated before Ser-64 (10, 12). This model is based on the finding that mutation of any one of the three TP sites in PHAS-I markedly decreased the phosphorylation of Ser-64 in PHAS-I overexpressed in HEK293 cells. The loss of Ser-64 phosphorylation as a result of these mutations was confirmed with the Ser(P)-64 antibodies (Fig. 2*B*). More importantly, results with these antibodies provide independent evidence of ordered phosphorylation and indicate that it occurs not only with overexpressed PHAS-I, as in previous studies (10, 12), but also with endogenous PHAS-I. Ser-64 phosphorylation does not appreciably affect mobility unless Thr-69 is also phosphorylated, in which case β is converted to γ (10). Ser(P)-64 reactivity in PHAS-I from both transfected HEK293 cells (Fig. 2*B*) and 3T3-L1 adipocytes (Fig. 5*A*) was observed exclusively in the γ form, supporting the conclusion that phosphorylation of other sites must occur before phosphate accumulates in Ser-64.

The ordered mechanism has hindered attempts to determine which phosphorylation sites directly control the dissociation of the PHAS-I·eIF4E complex in cells, as mutation of any one of the three TP sites blocks Ser-64 phosphorylation (10). *In vitro* phosphorylation of either Thr-45 or Ser-64 is sufficient to block binding to eIF4E, whereas phosphorylation of either Thr-36 or Ser-82 has relatively little effect on binding (10, 11). It has not been feasible to determine whether phosphorylation of Thr-69 *in vitro* directly inhibits eIF4E binding because of our inability

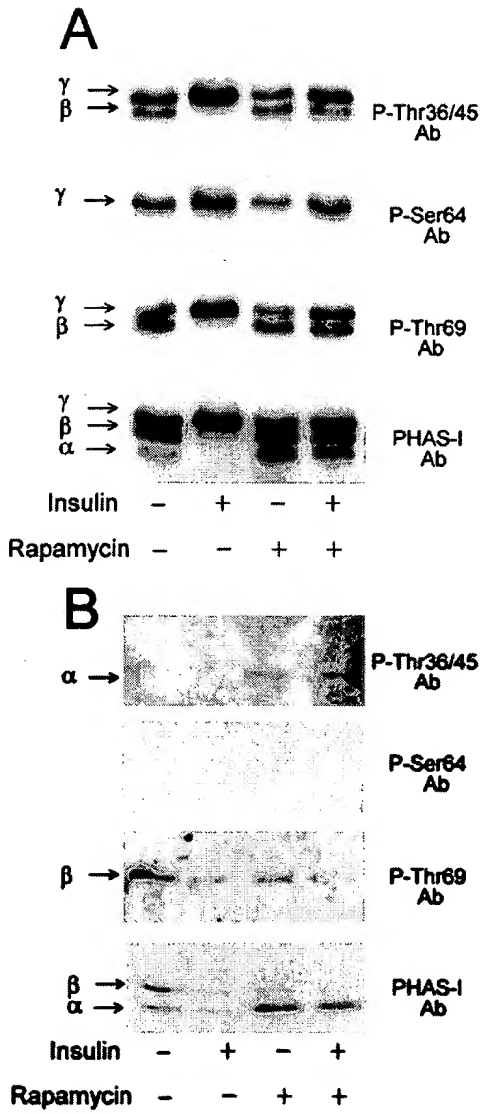

FIG. 5. **Phospho-specific antibody immunoblots of PHAS-I from 3T3-L1 adipocytes incubated with insulin and rapamycin.** *A*, immunoblots were prepared as described in the legend to Fig. 4. *B*, PHAS-I bound to eIF4E was isolated from extracts of 3T3-L1 adipocytes by using m^7GTP-Sepharose. Samples were subjected to SDS-PAGE, and immunoblots were prepared with the antibodies described in the legend to Fig. 4.

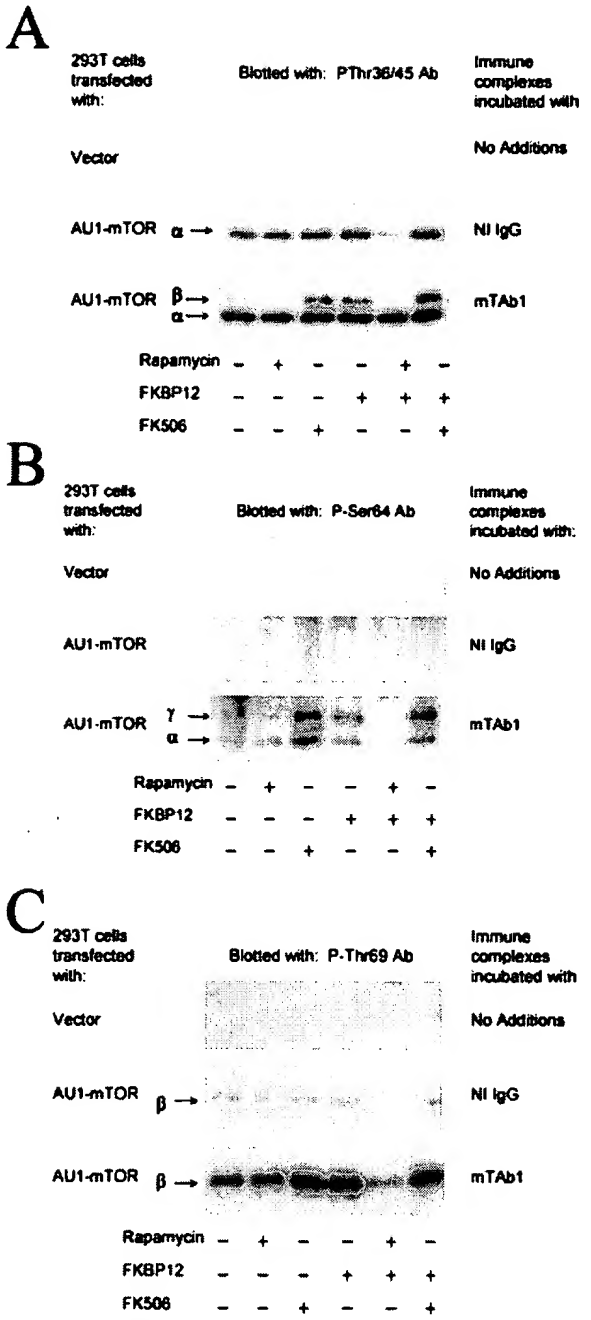

FIG. 6. **Reactivity of phospho-specific antibodies with PHAS-I phosphorylated by AU1-mTOR *in vitro*.** HEK293T cells were transfected with vector alone or with pcDNA-3 containing AU1-mTOR. After 18 h extracts were prepared, and immunoprecipitations were conducted with AU1 antibody. Immune complexes were incubated at 22 °C without additions, with nonimmune IgG (*NI IgG*), or with mTAb1. Rapamycin (10 μM), FKBP12 (10 μM), or FK506 (10 μM) were present as indicated. After 90 min kinase reactions were initiated by adding buffer containing Mn^{2+}, ATP, and PHAS-I. The reactions were terminated after incubating at 30 °C for 120 min, and samples were subjected to SDS-PAGE. Immunoblots were prepared with Thr(P)-36/45 antibodies (*A*), Ser(P)-64 antibodies (*B*), or Thr(P)-69 antibodies (*C*).

to phosphorylate selectively this site to a sufficiently high stoichiometry by using available kinases. The reactivity with Thr(P)-69 antibodies demonstrates the β form of PHAS-I that copurifies with eIF4E is phosphorylated in Thr-69 (Fig. 5*B*). Therefore, it is clear that phosphorylation of Thr-69 is not sufficient to abolish eIF4E binding. The eIF4E-bound α form reacted with Thr(P)-36/45 antibodies (Fig. 5*B*). This result was anticipated from previous peptide mapping studies, which determined that Thr-36 was the only phosphorylated site in the α form from PHAS-I bound to eIF4E after rapamycin treatment of rat adipocytes (9). Whereas phosphorylation of neither Thr-36 nor Thr-69 alone is sufficient to promote dissociation of the PHAS-I·eIF4E complex, these two sites may contribute indirectly to the control of binding by allowing phosphorylation of Ser-64 (10, 12). Ser(P)-64 antibody did not recognize the eIF4E-bound fraction of PHAS-I. Moreover, the γ form of PHAS-I, which we have now shown contains all of the phosphorylated Ser-64, was never found in the eIF4E-bound fraction (Fig. 5*B*). Therefore, it seems clear that PHAS-I does not bind to eIF4E when Ser-64 is phosphorylated in cells.

The reactivities of all three phospho-specific antibodies were increased by insulin and amino acids (Fig. 3, *A–C*), providing independent confirmation of the previous conclusion that the two treatments increased the phosphorylation of the same sites

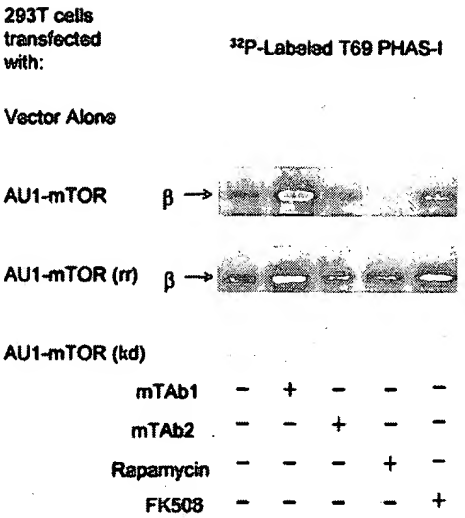

FIG. 7. **Phosphorylation of T69 PHAS-I by AU1-mTOR proteins *in vitro*.** HEK293T cells were transfected with vector alone or with pcDNA-3 vectors containing AU1-mTOR, AU1-mTOR (rr), or AU1-mTOR (kd). After 18 h extracts were prepared, and immunoprecipitations were conducted with AU1 antibody. Immune complexes were incubated at 22 °C with the following: no additions, mTAb1, mTAb2, rapamycin plus FKBP12, or FK506 plus FKBP12. After 90 min kinase reactions were initiated by adding buffer containing Mn^{2+}, [γ-^{32}P]ATP, and T69 PHAS-I. The reactions were terminated after incubating at 30 °C for 120 min, and samples were subjected to SDS-PAGE. Autoradiograms showing ^{32}P-labeled T69 PHAS-I are presented.

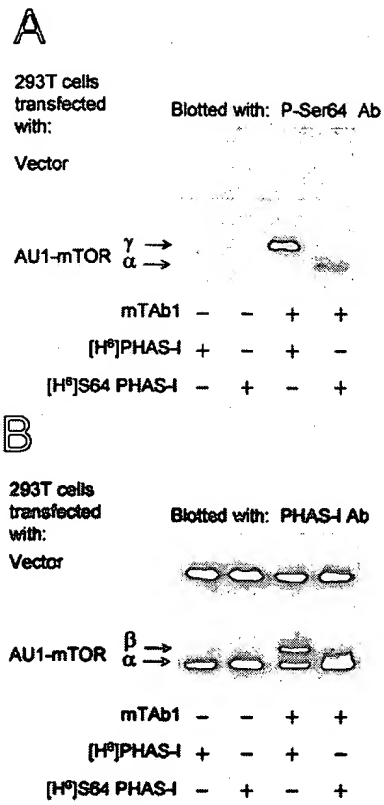

FIG. 8. **Mutation of the TP sites decreases phosphorylation of Ser-64 by AU1-mTOR *in vitro*.** HEK293T cells were transfected with vector alone or with pcDNA-3 containing AU1-mTOR. After 18 h extracts were prepared, and immunoprecipitations were conducted with AU1 antibody. Immune complexes were incubated at 22 °C without additions or with mTAb1. After 90 min kinase reactions were initiated by adding buffer containing Mn^{2+}, ATP, and either [His^6]PHAS-I or [His^6]S64 PHAS-I. The reactions were terminated after incubating at 30 °C for 120 min, and samples were subjected to SDS-PAGE. Immunoblots were prepared with Ser(P)-64 antibodies (*A*) or PHAS-I antibodies (*B*).

in PHAS-I (10). These results are consistent with the interpretation that insulin and amino acids signal via a common pathway. The finding that the effects of the two stimuli are attenuated by rapamycin implicates mTOR. The ability of mTOR to phosphorylate PHAS-I *in vitro* is increased after either HEK293 cells or 3T3-L1 adipocytes are incubated with insulin (28), and one hypothesis is that mTOR directly phosphorylates PHAS-I in cells (12, 16, 17). There is general agreement that mTOR phosphorylates Thr-36 and Thr-45 *in vitro* (11, 12, 17, 19), but some have argued that mTOR does not phosphorylate Ser-64 and Thr-69 (12, 17), which paradoxically are the most rapamycin-sensitive sites in HEK293 cells (10) (Fig. 3). In the present study phosphorylation was essentially confined to Thr-36 and Thr-45 when mTOR was incubated without mTAb1; however, the phosphorylation of both Ser-64 (Fig. 6*B*) and Thr-69 (Fig. 6*C*) was dramatically increased when AU1-mTOR immune complexes were incubated with mTAb1. The most straightforward explanation is that the activation of mTOR, as occurs in response to mTAb1 *in vitro* or other stimuli in cells, is necessary for phosphorylation of Ser-64 and Thr-69 by mTOR.

Recently, Sekulic *et al.* (29) have shown that deleting the mTAb1 epitope increases mTOR activity severalfold. Thus, the mTAb1 epitope, which is located near the COOH terminus of mTOR, forms part of an inhibitory regulatory domain. Presumably, binding of mTAb1 activates mTOR by interfering with the inhibitory function of this domain. Interestingly, Ser-2448, which is located in the domain, is phosphorylated by protein kinase B *in vitro* (29, 30), and phosphorylation of this site in cells is correlated with an increase in the PHAS-I kinase activity of mTOR (28, 29).

One of the concerns with immune complex kinase assays is that the activity observed might be due to another enzyme, either associated with or contaminating the immunopurified preparation. The activity that we have described does not appear to be due to the Ser/Thr kinases that have been reported to associate with mTOR. An antiserum generated by immunizing rabbits with a peptide identical to that used to make mTAb2 was reported to release a Ser-64 kinase from mTOR immunoprecipitates (31). This enzyme was reported to phosphorylate specifically Ser-64, to prefer Mg^{2+} to Mn^{2+}, and to phosphorylate PHAS-I only when it was bound to eIF4E. Based on these properties, the "mTOR-associated" kinase could not account for the observed phosphorylation of Ser-64 in the present experiments, which were performed with Mn^{2+} using the free PHAS-I protein as substrate. Moreover, we have been unable to reproduce the release of Ser-64 kinase with mTAb2. However, it is not uncommon for the specificities of antibodies to differ, even among animals immunized with the same antigen. Therefore, the recovery of the Ser-64 kinase may require the use of a particular antiserum. Protein kinase Cδ has been recently reported to associate with mTOR (32), and the activity of this kinase is inhibited by rapamycin treatment of cells (33). In preliminary experiments, we found that recombinant protein kinase Cδ phosphorylated PHAS-I but not in sites that react with any of the phospho-specific antibodies.

The fact that adding mTAb1 to the immunopurified AU1-mTOR increased the phosphorylation of Ser-64 and Thr-69 (Fig. 6, *B* and *C*) supports the argument that mTOR phosphorylates these two sites. When considered with other evidence, a strong case can be made for this conclusion. Immune complexes were washed under conditions at least as stringent as in other studies of mTOR kinase specificity (12), and the phosphoryla-

tion of both Ser-64 and Thr-69 was dependent on expression of the recombinant AU1-mTOR (Fig. 6, *B* and *C*). No phosphorylation was detected when immunoprecipitations with AU1-antibody were conducted using samples from cells transfected with vector alone, and rapamycin plus FKBP12 inhibited the phosphorylation of both Ser-64 and Thr-69 in PHAS-I (Fig. 6, *B* and *C*). In contrast, mTOR having a Ser-2035 → Ile point mutation that decreases affinity for rapamycin-FKBP12 phosphorylated Thr-69 and Ser-64,[2] but its activity was resistant to inhibition by rapamycin-FKBP12. mTOR rendered kinase-dead by an Asp-2338 → Ala point mutation in the kinase domain did not phosphorylate T69 PHAS-I, indicating that the phosphorylation required a functional mTOR kinase (Fig. 7).

If it is assumed that mTOR phosphorylates all of the sites in PHAS-I in cells except Ser-82, then rapamycin treatment would be expected to decrease the overall phosphorylation of the protein, consistent with the effects observed in the present study. Moreover, if prior phosphorylation of Thr-36 and Thr-45 facilitated the phosphorylation of Ser-64 and Thr-69, as has been proposed in the ordered model (12), then the phosphorylation of the latter two sites would be expected to be more sensitive to rapamycin. This is because phosphorylation of Thr-69 and Ser-64 would be reduced, not only through direct reduction of mTOR activity but also by decreases in the amount of phosphorylated Thr-36 and Thr-45. The phosphorylation of the TP sites in PHAS-I appeared to facilitate the phosphorylation of Ser-64 by AU1-mTOR *in vitro* (Fig. 8*A*), and the phosphorylation of Ser-64 and Thr-69 by mTOR *in vitro* was more sensitive to rapamycin (Fig. 6, *A–C*). Thus, although the issue of whether mTOR phosphorylates PHAS-I *in vivo* has not been resolved, there are intriguing parallels between the phosphorylation of PHAS-I by mTOR *in vitro* and the rapamycin-sensitive phosphorylation of PHAS-I in cells.

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